British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01381.x
www.brjpharmacol.org

Themed Section: Cannabinoids in Biology and Medicine, Part II

RESEARCH PAPER

A new cannabinoid CB_2 receptor agonist HU-910 attenuates oxidative stress, inflammation and cell death associated with hepatic ischaemia/reperfusion injury

Bèla Horváth[1,5]*, Lital Magid[3]*, Partha Mukhopadhyay[1]*, Sándor Bátkai[1], Mohanraj Rajesh[1], Ogyi Park[2], Galin Tanchian[1], Rachel Y Gao[1], Catherine E Goodfellow[4], Michelle Glass[4], Raphael Mechoulam[2] and Pál Pacher[1]

[1]*Laboratory of Physiologic Studies and* [2]*Liver Disease, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, Maryland, USA,* [3]*Institute of Drug Research, Faculty of Medicine, Hebrew University of Jerusalem, Ein Kerem, Jerusalem, Israel,* [4]*Department of Pharmacology and Clinical Pharmacology, Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand, and* [5]*Institute of Human Physiology and Clinical Experimental Research, Semmelweis University, Budapest, Hungary*

Correspondence
Pál Pacher, Section on Oxidative Stress Tissue Injury, Laboratory of Physiological Studies, National Institutes of Health/NIAAA, 5625 Fishers Lane, MSC-9413, Bethesda, MD 20892-9413, USA.
E-mail: pacher@mail.nih.gov

*Equally contributed.

Keywords
cannabinoids; oxidative stress; inflammation; ischaemia-reperfusion

Received
29 November 2010
Revised
27 February 2011
Accepted
15 March 2011

BACKGROUND AND PURPOSE

Cannabinoid CB_2 receptor activation has been reported to attenuate myocardial, cerebral and hepatic ischaemia-reperfusion (I/R) injury.

EXPERIMENTAL APPROACH

We have investigated the effects of a novel CB_2 receptor agonist ((1S,4R)-2-(2,6-dimethoxy-4-(2-methyloctan-2-yl)phenyl)-7,7-dimethylbicyclo[2.2.1]hept-2-en-1-yl)methanol (HU-910) on liver injury induced by 1 h of ischaemia followed by 2, 6 or 24 h of reperfusion, using a well-established mouse model of segmental hepatic I/R.

KEY RESULTS

Displacement of [^{3}H]CP55940 by HU-910 from specific binding sites in CHO cell membranes transfected with human CB_2 or CB_1 receptors ($hCB_{1/2}$) yielded K_i values of 6 nM and 1.4 μM respectively. HU-910 inhibited forskolin-stimulated cyclic AMP production by hCB_2 CHO cells (EC_{50} = 162 nM) and yielded EC_{50} of 26.4 nM in [^{35}S]GTPγS binding assays using hCB_2 expressing CHO membranes. HU-910 given before ischaemia significantly attenuated levels of I/R-induced hepatic pro-inflammatory chemokines (CCL3 and CXCL2), TNF-α, inter-cellular adhesion molecule-1, neutrophil infiltration, oxidative stress and cell death. Some of the beneficial effect of HU-910 also persisted when given at the beginning of the reperfusion or 1 h after the ischaemic episode. Furthermore, HU-910 attenuated the bacterial endotoxin-triggered TNF-α production in isolated Kupffer cells and expression of adhesion molecules in primary human liver sinusoidal endothelial cells stimulated with TNF-α. Pretreatment with a CB_2 receptor antagonist attenuated the protective effects of HU-910, while pretreatment with a CB_1 antagonist tended to enhance them.

CONCLUSION AND IMPLICATIONS

HU-910 is a potent CB_2 receptor agonist which may exert protective effects in various diseases associated with inflammation and tissue injury.

LINKED ARTICLES

This article is part of a themed section on Cannabinoids in Biology and Medicine. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2012.165.issue-8. To view Part I of Cannabinoids in Biology and Medicine visit http://dx.doi.org/10.1111/bph.2011.163.issue-7

Abbreviations

4-HNE, 4-hydroxy-2-nonenal (marker of lipid peroxidation); CB_2 or CB_1 receptor, cannabinoid 1 or 2 receptor; HU-910, ((1S,4R)-2-(2,6-dimethoxy-4-(2-methyloctan-2-yl)phenyl)-7,7-dimethylbicyclo[2.2.1]hept-2-en-1-yl)methanol; I/R, ischaemia/reperfusion; ICAM-1, inter-cellular adhesion molecule 1, CD54;CCL2, monocyte chemotactic protein-1; CCL3, macrophage inflammatory protein-1α; CXCL2, macrophage inflammatory protein-2α; VCAM-1, vascular cell adhesion molecule 1

Introduction

Ischaemia followed by reperfusion (I/R) is a major mechanism leading to end-organ damage which complicates the course of circulatory shock, organ transplantation, myocardial infarction, stroke and may accompany surgical interventions involving vascular occlusion. The common pathology of these conditions is that the transient disruption of the normal blood supply to target organs followed by reperfusion induces an acute generation of reactive oxygen and nitrogen species subsequent to reoxygenation upon vascular reopening (Ferdinandy and Schulz, 2003; Pacher *et al.*, 2007). These changes initiate a chain of deleterious cellular responses leading to inflammation, cell death and eventually culminating in target organ dysfunction or failure (Liaudet *et al.*, 2003; Pacher and Hasko, 2008).

Activation of cannabinoid CB_2 receptors (nomenclature follows Alexander *et al.*, 2011) has been reported to attenuate injury in preclinical models of myocardial (Montecucco *et al.*, 2009), cerebral (Zhang *et al.*, 2009a,b) and hepatic I/R injury (Batkai *et al.*, 2007; Rajesh *et al.*, 2007) in addition to numerous other inflammatory disorders (Pacher *et al.*, 2006; Di Marzo, 2008; Pacher and Mechoulam, 2011). Furthermore, CB_2 receptors protect liver against development of fibrosis (Julien *et al.*, 2005; Lotersztajn *et al.*, 2008) and may also play an important role in liver regeneration (Teixeira-Clerc *et al.*, 2010) and protection against alcohol-induced liver injury.

We have already demonstrated that the CB_2 receptor agonists HU-308 and JWH-133 afforded protection against hepatic I/R injury only in doses of 10 and 20 $mg \cdot kg^{-1}$ (given i.p.) (Batkai *et al.*, 2007; Rajesh *et al.*, 2007). In this study, we have investigated the effects of a novel CB_2 receptor agonist ((1S,4R)-2-(2,6-dimethoxy-4-(2-methyloctan-2-yl)phenyl)-7,7-dimethylbicyclo[2.2.1]hept-2-en-1-yl)methanol (HU-910; Figure 1) with greater *in vivo* efficacy (effective from 1 $mg \cdot kg^{-1}$ i.p.) compared with HU-308 and JWH-133, on liver injury induced by 1 h of ischaemia followed by 2, 6 or 24 h of reperfusion, using a well-established mouse model of segmental hepatic I/R (Rajesh *et al.*, 2007; Moon *et al.*, 2008; Abe *et al.*, 2009; Mukhopadhyay *et al.*, 2011b). We have also explored the effects of HU-910 on the production of TNF-α by murine Kupffer cells (key resident macrophage-derived inflammatory cells of the liver) triggered by bacterial endotoxin/lipopolysaccharide (LPS), and on the expression of adhesion molecules in primary human liver sinusoidal endothelial cells (HLSEC) stimulated with TNF-α.

Our findings strengthen the potential of HU-910 for the prevention or treatment of I/R injury and other inflammatory disorders.

Methods

Cell line generation and maintenance

The cDNA clones for human CB_1 and CB_2 receptors tagged with three haemagglutinin (HA) sequences were obtained from the Missouri S&T cDNA Resource Center (http://www.cdna.org) in cloning vector pcDNA3.1 + (pcDNA 3 × HA $hCB_{1/2}$). The vector containing the human CB_2 receptor was transfected directly into CHO-KI cells obtained from ATCC. The HA-tagged human CB_1 receptor sequence was subcloned into the pef4-V5-HisA vector with Kpn1 (Roche) and Pme1 (New England Biolabs) restriction enzymes and subsequently transfected into CHO-K1 cells using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Cells were clonally isolated by limited dilution and screened by immunocytochemistry for expression of the HA tag. Clones expressing the HA tag were also screened by reverse transcription PCR to confirm expression of human CB_1 and human CB_2 receptor mRNA transcripts.

Cells were maintained in Dulbecco's modified Eagle medium: nutrient mixture F-12 (DMEM/F12) media supplemented with 10% fetal bovine serum (FBS), 100 $units \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin and 2 mM L-glutamine. Transfected cell lines were maintained with additional 250 $\mu g \cdot mL^{-1}$ zeocin for CHO-CB_1 transfected cells and 500 $\mu g \cdot mL^{-1}$ G-418 for CHO-CB_2 transfected cells (all reagents obtained from Invitrogen).

Membrane preparation

Cells were grown to 90–100% confluence and harvested in ice-cold phosphate buffered saline with 5 mM EDTA. Cells were centrifuged at 200× *g* for 10 min and frozen at –80°C until required. Cell pellets were thawed with cold 0.32 M sucrose and homogenized with a glass homogenizer. The homogenate was centrifuged at 1000× *g* for 10 min at 4°C and the supernatant centrifuged in a Sorvall ultracentrifuge for 30 min at 100 000× *g*. The pellet was then washed in ice-cold Tris wash buffer and re- centrifuged twice more. The final pellet was resuspended in 50 mM Tris pH 7.5, 0.5 mM EDTA. Protein concentration was determined using the Dc protein assay kit (Bio-Rad, Hercules, CA, USA).

Competition binding assay

The K_d of CP 55,940 in the isolated CB_1 and CB_2 receptor expressing membranes was previously determined to be 2.3 nM and 1.5 nM respectively. Competition binding assays at 2.5 nM [^{3}H]-CP 55,940 (PerkinElmer) were carried out to determine the K_i values for tested compounds. Membranes (5–10 μg) were incubated with radioligand and a range of concentrations of test compounds in binding buffer (50 mM Tris pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA) with 0.5% (w/v) bovine serum albumin (BSA) (ICP Bio, New Zealand), at 30°C for 60 min. Stock solutions of putative cannabinoid ligands were prepared in DMSO to a concentration of 10 mM. Six different final concentrations of compounds were used ranging from 0.1 nM to 50 μM. Non-specific binding was determined in the presence of 1 μM non-radioactive CP 55,940 (Tocris Cookson). Assays were terminated by addition of 3 mL ice-cold binding buffer and filtration through GF/C filters (Whatman) pre-soaked in cold binding buffer, followed by two washes in the same buffer.

Radioactivity was determined by incubation of filters with Irgasafe scintillation fluid (PerkinElmer) and scintillation counting in a Wallac Trilux using Microbeta Trilux software. Data was analysed using the Prism 4.02 program (GraphPad Software, San Deigo, CA, USA).

cAMP assay

CHO-CB_1 and CHO-CB_2 cells were seeded at a density of 10^4 cells per well in poly-L-lysine treated 96-well culture plates (BD Biosciences). The following day wells were incubated with 40 μL DMEM/F12 containing 0.5% (w/v) BSA and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich) for 30 min prior to 15 min stimulation with 50 μM forskolin (Tocris Cookson) and varying concentrations of indicated compounds at 37°C, 5% CO_2. Assays were stopped by removal of media and addition of 100% ice-cold ethanol. Plates were then frozen for a minimum of 2 h before complete evaporation of ethanol. The well contents were then reconstituted in 50 μL cAMP assay buffer (20 mM HEPES pH 7.5 and 5 mM EDTA). Half of the reconstituted sample was transferred to round bottom 96-well plates (Greiner Bio-One GmbH) with 50 μL 0.01% w/v cAMP dependent protein kinase A [PKA (Sigma-Aldrich) in 1 mM sodium citrate pH 6.5 with 2 mM dithiothreitol] and 25 μL [^{3}H]-cAMP (at 22 nM in cAMP assay buffer) (GE Healthcare, Life Sciences). Samples were then allowed to equilibrate for 3–18 h. Following this, a charcoal slurry [5% (w/v) activated charcoal and 0.2% (w/v) BSA in cAMP assay buffer] was added to the samples and the plates centrifuged at 3000× *g*, 4°C for 5 min. A sample of the supernatant was then transferred to 96-well flexible microplates (PerkinElmer) and 200 μL Irgasafe scintillation fluid (PerkinElmer) added. Plates were sealed, vigourously agitated and scintillation counting performed by a Wallac Trilux using Microbeta Trilux software.

[^{35}S]GTPγS binding assay

Human CB_2 receptor expressing CHO-K1 membranes (5 μg per incubation mixture) were diluted in 50 mM Tris-HCl (pH 7.5) and 0.5 mM EDTA and added to the indicated compounds in a pre-mixed incubation cocktail. Final incubation concentrations were 55 mM Tris-HCl (pH 7.4), 1 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$, 0.5% BSA, 50 μM GDP, 0.2 nM [^{35}S]GTPγS (PerkinElmer) with varied concentrations of compounds (0.1 nM–10 μM) and 5 μg membrane. Incubations were continued for 60 min at 30°C in a shaking water bath. Assays were terminated by addition of 2 mL ice-cold wash buffer (50 mM Tris-HCl, pH 7.5 and 5 mM $MgCl_2$) and filtration through pre-soaked GF/C filters (Whatman), followed by two further washes. Radioactivity was determined as described for competition binding assays.

Hepatic ischaemia-reperfusion

All animal care and experimental procedures complied with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committees of the National Institute on Drug Abuse (NIAAA). Male C57BL/6J mice (25–30 g; Jackson Laboratories, Bar Harbor, ME, USA) were anesthetized with pentobarbital sodium (65 mg·kg^{-1} i.p.). We used the model of segmental (70%) hepatic ischaemia, as described by Mukhopadhyay *et al.*, (2011b). Briefly, the liver was exposed by midline laparotomy and the hepatic artery and the portal vein were clamped using an atraumatic micro-serrefine. This method of partial ischaemia prevents mesenteric venous congestion by allowing portal decompression throughout the right and caudate lobes of the liver. The duration of hepatic ischaemia was 60 min, after which the vascular clips were removed and liver was reperfused for 2, 6 or 24 h, as indicated. Sham surgeries were identical except that hepatic blood vessels were not clamped with a micro-serrefine. The liver was kept moist at 37°C with gauze soaked in 0.9% saline. Body temperature was monitored with a rectal temperature probe and was maintained at 37°C by a heating blanket. Treatments with HU-910 0.3, 1, 3 and 10 mg·kg^{-1} or vehicle (i.p.), started 2 h before I/R or were given after ischaemia at the moment of reperfusion, as indicated in the text. CB_1 and CB_2 receptor antagonists were given 2 h before ischaemia or HU-910 treatment. At the experimental end points, blood was collected and liver samples were removed and snap-frozen in liquid nitrogen for determining biochemical parameters or fixed in 4% buffered formalin for histopathological evaluation (Moon *et al.*, 2008).

Serum aspartate amino-transferase (AST) and alanine amino-transferase (ALT) levels

The activities of AST and ALT, indicators of liver cellular damage (necrosis), were measured in serum samples using a clinical chemistry analyser system (VetTest 8008, IDEXX laboratories, Westbrook, ME, USA) (Moon *et al.*, 2008).

Histological examination of liver sections

Liver samples were fixed in 4% buffered formalin. After embedding and cutting 5 μm slices, all sections were stained with haematoxylin/eosin. Neutrophils were stained for myeloperoxidase (MPO) using anti-MPO antibody, according to the manufacturer's protocol (Biocare Medical, Concord, CA, USA), and samples were counter-stained with nuclear fast red as described (Mukhopadhyay *et al.*, 2011b). Histological evaluation was performed without knowledge of the treatments.

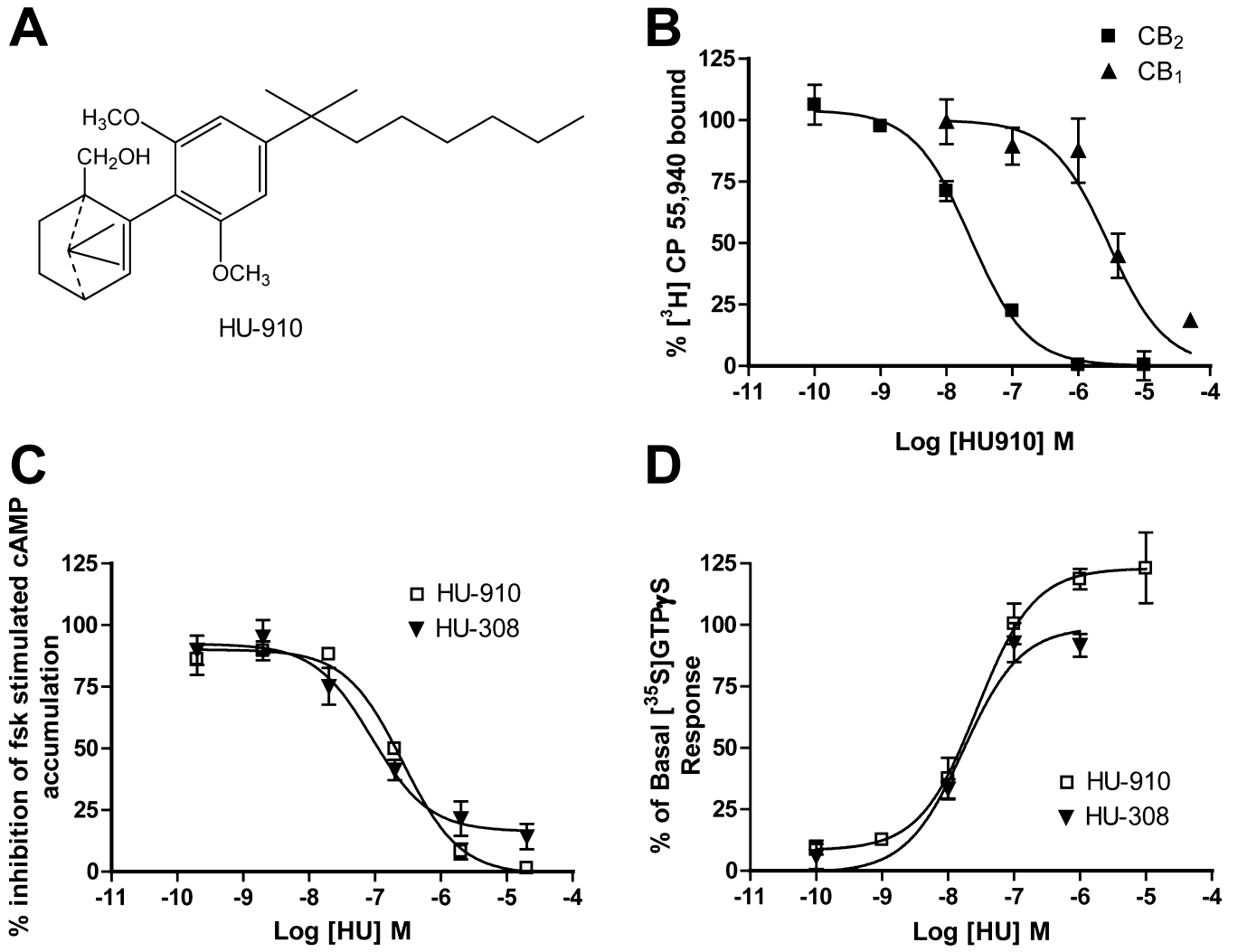

Figure 1

Chemical structure and representative graphs of binding assays of HU-910. (A) Chemical structure of HU-910. (B) Competition binding assays were performed with either CHO-CB_1 or CHO-CB_2 cellular membranes by displacement of 2.5 nM [^{3}H]CP 55,940. (C) Representative graph of inhibition of forskolin (fsk)-stimulated cAMP accumulation in CHO 3HA hCB_2 cells by HU910. Data was normalized to the maximum response achieved by HU-308 in parallel stimulations. (D) GTPγS binding at CB_2. HU910 mediated GTPγS binding at CHO-CB_2 membranes. Data is shown as [^{35}S]GTPγS binding normalized to maximal HU-308 binding under the same experimental conditions. Values in graphs are means ± SEM, n = 4–5.

Real-time PCR analyses of mRNA

Total RNA was isolated from liver homogenate using TRIzol reagents (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. The isolated RNA was treated with RNase-free DNase (Ambion, Austin, TX, USA) to remove traces of genomic DNA contamination. Samples (1μg) of total RNA were reverse-transcribed to cDNA using the SuperScript II (Invitrogen, Carlsbad, CA, USA). The target gene expression was quantified with Power SYBER Green PCR Master Mix using an ABI HT7900 real-time PCR instrument (Applied Biosystems, Foster City, CA, USA). Each amplified sample in all wells was analysed for homogeneity using dissociation curve analysis. After denaturation at 95°C for 2 min, 40 cycles were performed at 95°C for 10 s and at 60°C for 30 s. Relative quantification was calculated using the comparative CT method (2-ΔΔCt method: ΔΔCt = ΔCt sample – ΔCt reference). Lower ΔCT values and lower ΔΔCT reflect a relatively higher amount of gene transcript.

Primers used were as follows:

CCL3, 5′-TGCCCTTGCTGTTCTTCTCTG-3′ and 5′-CAA CGATGAATTGGCGTGG-3′; CXCL2, 5′-AGTGAACTGCGCTG TCAATGC-3′ and 5′-AGGCAAACTTTTTGACCGCC-3′; TNF-α, 5′-AAGCCTGTAGCCCACGTCGTA-3′ and 5′-AGGTACAACC CATCGGCTGG-3′; ICAM-1, 5′-AACTTTTCAGCTCCGGTC CTG-3′ and 5′-TCAGTGTGAATTGGACCTGCG-3′; CB_1, 5′-ATGAAGTCGATCCTAGATGGCCTTGCAGA-3′ and 5′TCA CAGAGCCTCGGCAGACGTG-3′; CB_2, 5′-GACCTTCACAGCC TCTGTGGGTA-3 and 5′-GATTTTCCCATCAGCCTCTGTCT-3; and actin, 5′-TGCACCACCAACTGCTTAG-3′ and 5′-GGATGC AGGGATGATGTTC-3′.

Hepatic 4-hydroxynonenal (HNE) content

Lipid peroxides are unstable indicators of oxidative stress in cells that decompose to form more complex and reactive compounds such as HNE, which has been shown to be capable of binding to proteins and forming stable HNE adducts. HNE in the hepatic tissues was determined using a kit (Cell Biolabs, CA, USA). In brief, BSA or hepatic tissue extracts (10 μg·mL^{-1}) are adsorbed onto a 96-well plate for 12 h at 4°C. HNE adducts present in the sample or standard were probed with anti-HNE antibody, followed by an horseradish peroxidase (HRP)-conjugated secondary antibody. The content of HNE-protein adducts in an experimental sample was determined by comparing with a standard curve (Mukhopadhyay *et al.*, 2010).

Detection of hepatic carbonyl adducts

Carbonyl content in liver tissues was determined by OxiSelect Protein Carbonyl ELISA Kit (Cell Biolabs, CA, USA) (Mukhopadhyay *et al.*, 2011a). In brief, BSA standards or protein samples (10 μg·mL^{-1}) were adsorbed onto a 96-well plate for 2 h at 37°C. The protein carbonyls present in the sample or standard were derivatized to DNP hydrazone and probed with an anti-DNP antibody, followed by an HRP-conjugated secondary antibody. The protein carbonyl content in unknown sample was determined by comparing with a standard curve, prepared from reduced and oxidized BSA standards.

Detection of apoptosis by caspase 3/7 activity assays

Caspase 3/7 activity in hepatic tissue lysate was measured using the Apo-One Homogenous Caspase-3/7 assay kit (Promega Corp., Madison, WI, USA). An aliquot of caspase reagent was added to each well and mixed on a plate shaker for 1 h at room temperature shielded from light, and the fluorescence was measured (Rajesh *et al.*, 2009; 2010).

Hepatic DNA fragmentation ELISA

The quantitative determinations of cytoplasmic histone-associated-DNA-fragmentation (mono and oligonucleosomes) due to cell death in liver homogenizates were measured using ELISA kit (Roche Diagnostics GmbH) (Mukhopadhyay *et al.*, 2009; Rajesh *et al.*, 2010).

Isolation and stimulation of hepatic Kupffer cells

Livers were perfused in deeply anaesthetized mice and the livers were excised for isolation of Kupffer cells. Hepatic cellular contents were released from the stroma by digestion with the perfusion medium (hepatocyte wash media) containing collagenase type 1 (Sigma, St. Louis, MO, USA) and 2% Penicillin and Streptomycin (Invitrogen, Carlsbad, CA, USA). Kupffer cells were isolated using Optiprep gradient. Kupffer cell fraction was aspirated and washed twice with RPMI 1640 medium. Homogenous Kupffer cells population was obtained by using negative selection (anti-CD146) LS column according to the manufacturer's instruction (Miltenyi Biotec, Auburn, CA, USA) as described earlier (Mukhopadhyay *et al.*, 2011b). After additional washes, Kupffer cells were plated on to 96-well or six-well plates in RPMI 1640 media, containing 10% FBS and penicillin-streptomycin, and

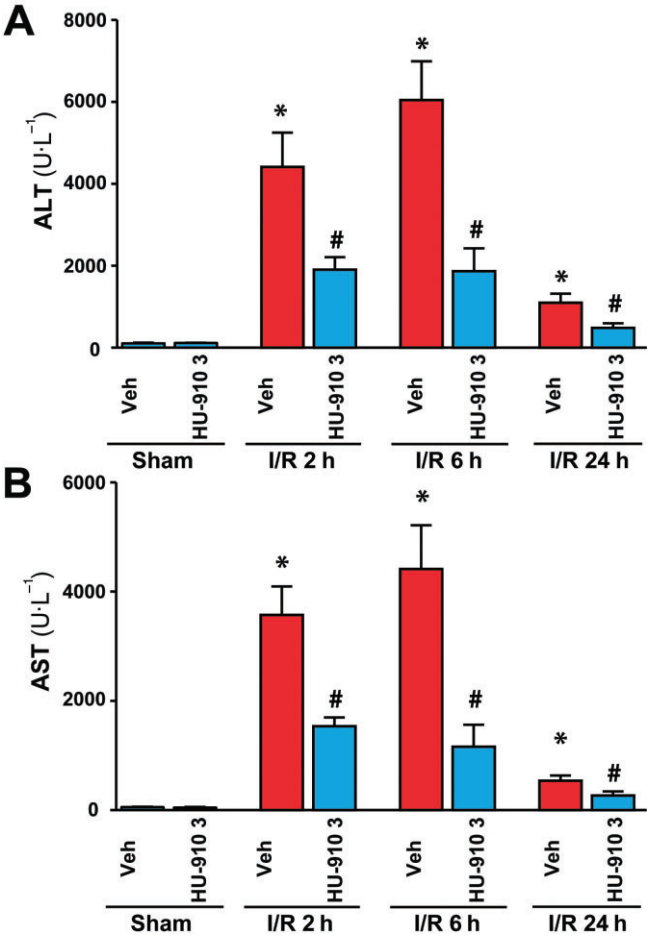

Figure 2

HU-910 attenuates hepatic ischaemia/reperfusion (I/R) injury. (A,B) Serum transaminase alanine amino-transferase (ALT) (A) and aspartate amino-transferase (AST) (B) levels in sham operated mice treated with vehicle (Veh) or HU-910 (n = 4–5) or in mice exposed to 1 h of hepatic ischaemia followed by 2, 6 and 24 h of reperfusion with vehicle or HU-910 (3 mg·kg^{-1}) given before ischaemia (n = 6–10). Results are mean ± SEM. *P < 0.05 vehicle versus I/R 2, 6 and 24 h; #P < 0.05 I/R 2, 6 and 24 h versus corresponding I/R + HU-910.

incubated overnight in a CO_2 incubator at 37°C and 5% CO_2. Cells were maintained for 2 h with RPMI 1640 medium containing FBS (2%). HU-910 or HU-308 or the CB_2 receptor antagonists (SR144528 or AM-630) at indicated concentrations were added 1 h prior to LPS treatment. Cells were treated for 6 h with LPS (*Escherichia coli* O127:BB catalogue # L3129, Sigma Chemicals, St Louis, MO, USA). After the end of treatments, culture supernatants were removed and snap frozen in liquid nitrogen and assayed for the TNF-α concentrations with the use of ELISA kit (Mouse TNF-α; catalogue # SMTA00; R&D Systems, Minneapolis, MN, USA) as described by Mukhopadhyay *et al.*, (2011b).

Cell surface inter-cellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) expression assay

Cell surface expression of ICAM-1 and VCAM-1 in the HLSEC was measured by *in situ* ELISA as described (Rajesh *et al.*, 2007).

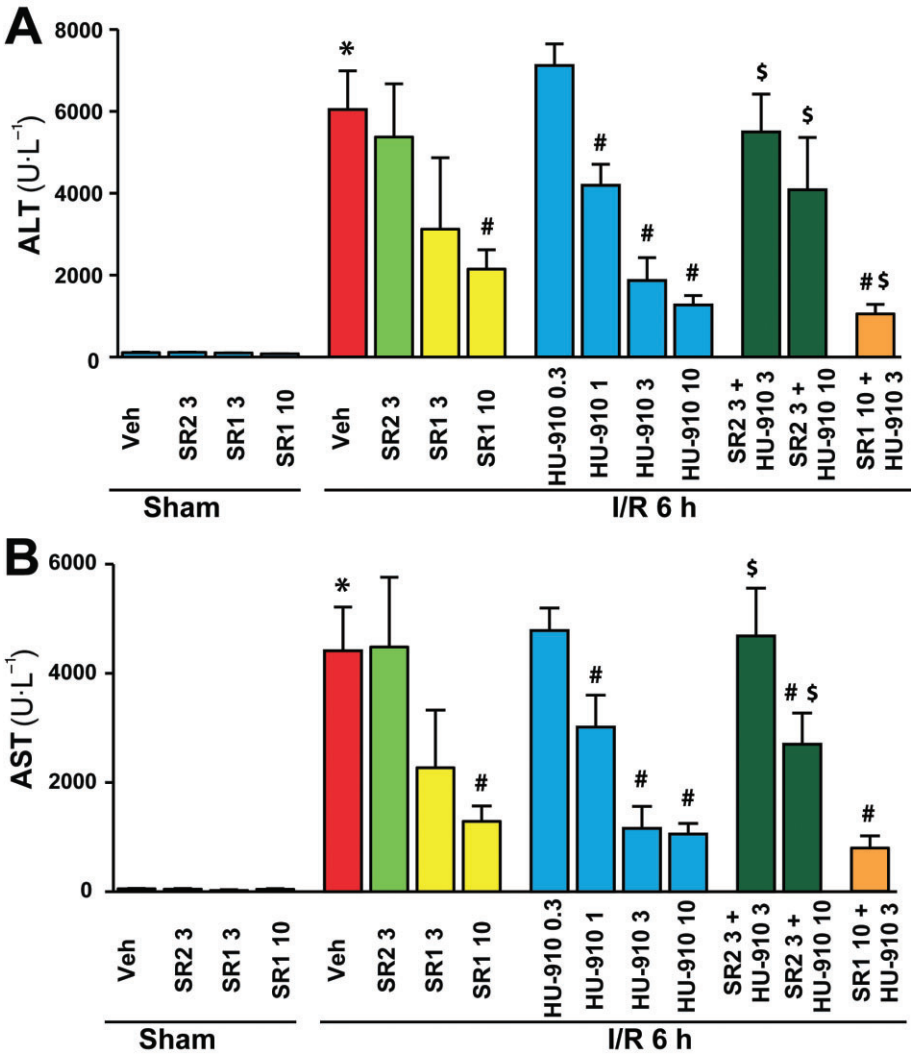

Figure 3

HU-910 dose-dependently attenuates hepatic ischaemia/reperfusion (I/R) injury: role of $CB_{1/2}$ receptors. (A,B) Serum transaminase alanine amino-transferase (ALT) (A) and aspartate amino-transferase (AST) (B) levels in sham operated mice treated with vehicle (Veh) or SR144528 (SR2; 3 mg·kg^{-1}) or SR141716A (SR1; 3 and 10 mg·kg^{-1}) (n = 4–5) or in mice exposed to 1 h of hepatic ischaemia followed by 6 h of reperfusion treated with vehicle or HU-910 (0.3, 1, 3 or 10 mg·kg^{-1}) or SR144528 (3 mg·kg^{-1}) or SR141716A (3 and 10 mg·kg^{-1}) or their combinations (n = 4–8) given before ischaemia. Results are mean ± SEM. *P < 0.05 vehicle versus I/R 6 h; #P < 0.05 I/R 6 h versus I/R 6 h + SR1/HU-910/HU-910 + SR2; $$P$ < 0.05 I/R 6 h + HU-910 (3 or 10 mg·kg^{-1}) versus corresponding I/R 6 h + SR2 + HU-910 or I/R 6 h + SR1 (10 mg·kg^{-1}) versus corresponding I/R 6 h + SR1 + HU-910 treatment.

In brief, HLSEC cells were grown in 96-well plates. After treatments as described in Figure 14, *in situ* ELISA was performed with anti-mouse ICAM-1 or VCAM-1 monoclonal antibodies (1:1500 dilution; R&D Systems) and by measuring the absorbance colorimetrically at 450 nm using the HRP-3,3′,5,5′-tetramethylbenzidine developing system (Sigma, St. Louis, MO, USA). Each treatment was performed in triplicate, and the experiments were repeated three times.

Analysis of data

For binding data the K_i was determined from IC_{50} values derived from competition binding data fitted with one site competition non-linear regression analysis by Prism 4.02 using the K_d values shown in Table 1. pIC_{50} values were determined from cAMP assays by fitting a sigmoidal concentration response curve. Results shown were generated by averaging at least three independently determined pIC_{50} values. Data shown is mean IC_{50} (95% confidence interval). Emax values were calculated as a percentage of the maximal response detected in parallel cAMP assays with HU-210 or HU-308 for CB_1 and CB_2 receptor expressing cells respectively. Data are displayed as the mean ± SEM.

For all experiments presented in Figures 2–14, values have been expressed as means and variability as SEM. Statistical

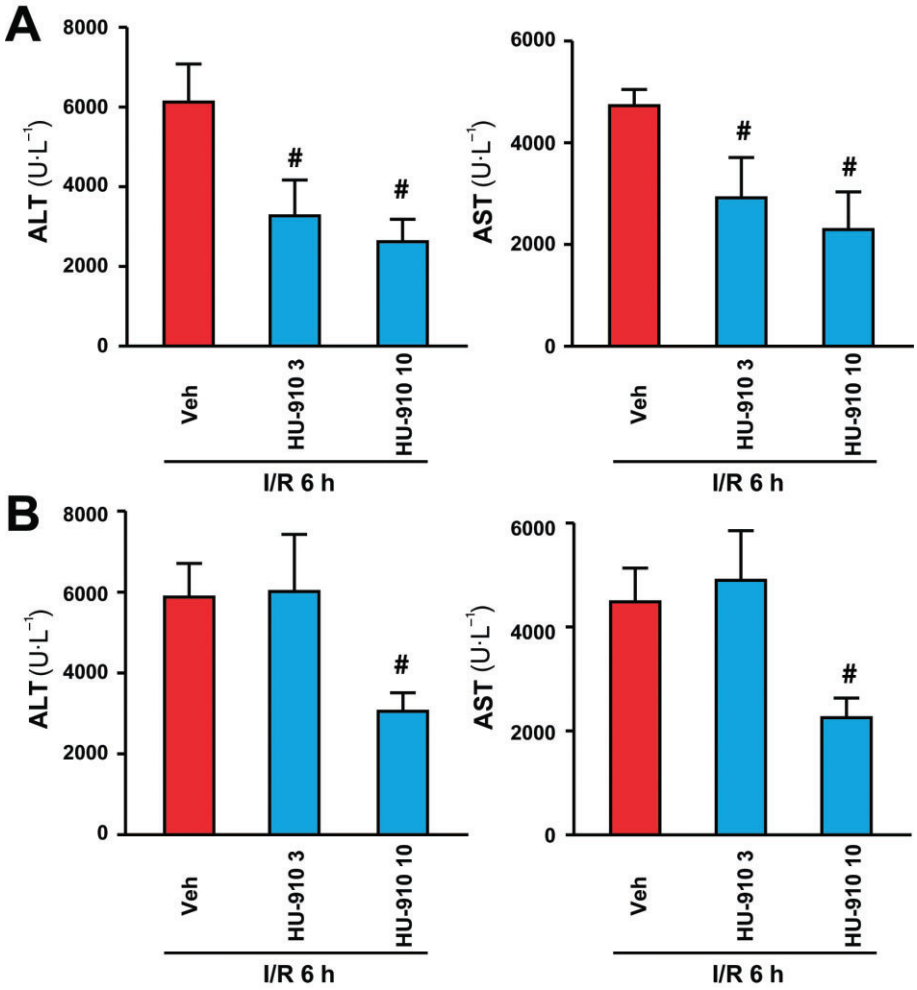

Figure 4

HU-910 treatment at reperfusion or 1 h later attenuates hepatic ischaemia/reperfusion (I/R) injury. Serum transaminase alanine amino-transferase (ALT) and aspartate amino-transferase (AST) levels in mice exposed to 1 h of hepatic ischaemia followed by 6 h of reperfusion treated with vehicle or HU-910 (3 or 10 mg·kg^{-1}) right after the ischaemia (A) or 1 h after reperfusion (B). Results are mean ± SEM, n = 7–8. #P < 0.05 versus corresponding mice exposed to I/R for 6 h.

Table 1

HU-910 activates CB_2 receptors *in vitro*

	Competition binding assay		cAMP assay				[^{35}S]GTPγS	
	CB_2 receptor	CB_1 receptor	CB_2 receptor		CB_1 receptor		CB_2 receptor	
Drug	K_i (nM)	K_i (μM)	IC_{50} (nM)	Emax (%)	IC_{50} (μM)	Emax	EC_{50} (nM)	Emax (%)
HU-910	6	1.37	162	105 ± 12	ND	–	26.4	121 ± 7
HU-308	14	ND	117	100 ± 0	ND	–	18.3	100 ± 0

Data shown are IC_{50} values. Emax values were calculated as a percentage of the maximal response detected in parallel cAMP assays with HU-308. Data are displayed as the mean ± SEM. ND, no detectable inhibition of cAMP or displacement of radioactive binding up to 10 μM.

significance among groups was determined by one-way ANOVA followed by Newman–Keuls *post hoc* analysis using GraphPad Prism 5 software (San Diego, CA, USA). Probability values of P < 0.05 were considered significant.

Materials

The chemical structure of HU-910 is shown in Figure 1. The synthesis of HU-910 has been presented at a meeting of the

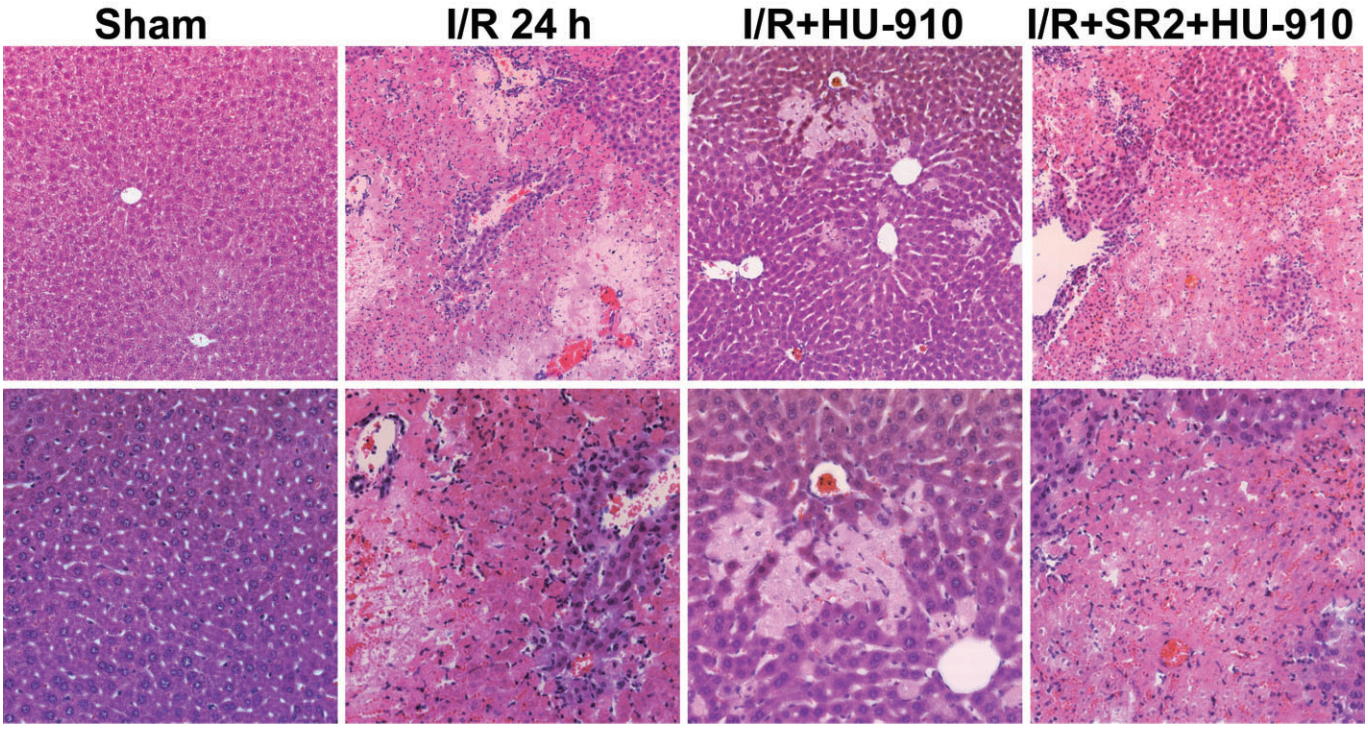

Figure 5

HU-910 attenuates histological damage at 24 h following ischaemia. Haematoxylin and eosin staining of representative liver sections of sham mice treated with vehicle (Sham), and mice exposed to 1 h of ischaemia followed by 24 h of reperfusion treated with vehicle [ischaemia/reperfusion (I/R) 24 h] or HU-910 3 mg·kg^{-1} (I/R + HU-910) or combined HU-910 3 mg·kg^{-1} and SR144528 (SR2; 3 mg·kg^{-1}) (I/R + SR2 + HU-910) given before ischaemia. Note the marked attenuation of the coagulation necrosis and inflammatory cell infiltration characteristic of I/R 24 h by HU-910, which was mostly prevented by pretreatment with SR144528. At this dose SR144528 alone had no effect on I/R-induced histopathological injury; likewise neither HU-910 nor SR144528 had effects on normal liver histology in sham controls (data not shown). Upper row of images depicts 200 × magnification, while the lower one 400 × magnification. A similar histological profile was seen in 3–5 livers per treatment group.

International Cannabinoid Research Society (Magid *et al.*, 2010a) and in a patent (Magid *et al.*, 2010b). HU-910 has a unique bicyclic structure, in the non-aromatic portion of the molecule. Its synthesis follows a number of steps starting with a Suzuki cross-coupling reaction using (+)-camphor-10-sulphonyl chloride (Sigma-Aldrich) and 2,6-dimethoxy-4-(2-methyloctan-2-yl)benzene (Dominianni *et al.*, 1977).

The CB_1 and CB_2 receptor antagonists/inverse agonists SR141716A and SR144528 were obtained from the National Institute on Drug Abuse (NIDA) Drug Supply Program. For *in vivo* administration, all drugs were dissolved in vehicle solution (one drop of Tween-80 in 3 mL 2.5% dimethyl sulphoxide in saline) as previously described (Batkai *et al.*, 2007). Vehicle solution was used in control experiments.

Results

As shown in Table 1 and in Figure 1B–D, HU-910 behaved in all assays as a potent agonist at human CB_2 receptors (comparable to the reference CB_2 receptor agonist HU-308).

HU-910 attenuates markers of hepatic I/R injury (ALT, AST)

For assessments of hepatocellular damage of the post-ischaemic liver, the serum transaminase activities (AST and ALT), markers of necrosis, were measured. After 1 h of ischaemia and a subsequent 2 or 6 h of reperfusion (I/R 2 h and I/R 6 h respectively), a dramatic increase in liver enzyme activities were observed in vehicle-treated C57Bl6/J mice as compared with sham-operated controls, which almost returned to baseline at 24 h of reperfusion (I/R 24 h) (Figure 2). HU-910 was able to attenuate these increases in the markers of hepatic I/R injury at all measured time points, while HU-910 alone had no effects on ALT and AST levels in the sham animals compared with the vehicle-treated group (Figure 2).

Because the peak elevation of ALT and AST is known to occur around 6 h of reperfusion (Abe *et al.*, 2009; Mukhopadhyay *et al.*, 2011b), as also demonstrated in Figure 2, this time point was chosen to evaluate the maximal hepatocellular injury (necrosis) in the subsequent experiments.

Pretreatment with HU-910 (0.3, 1, 3 or 10 mg·kg^{-1} i.p.) 2 h before the induction of the ischaemia dose-dependently attenuated the serum transaminase elevations (ALT and AST) at 6 h of reperfusion compared with vehicle (Figure 3). Furthermore, pretreatment with the CB_2 receptor antagonist SR144528 (3 mg·kg^{-1} i.p.) significantly attenuated the effect of 3 or 10 mg·kg^{-1} HU-910 at the time of the peak serum ALT and AST elevations, implying a role for CB_2 receptors in the beneficial effects of HU-910 (Figure 3). Because the protective effect of 3 and 10 mg·kg^{-1} of HU-910 were similar (Figure 3),

and the protection afforded by the lower dose could almost completely be prevented by pretreatment with SR144528 (3 mg·kg^{-1} i.p.), 3 mg·kg^{-1} HU-910 was used in the subsequent experiments. Given alone, SR144528 (3 mg·kg^{-1}) did not affect the I/R-induced increase in liver enzymes, while pretreatment with the CB_1 receptor antagonist SR141716A (3 mg·kg^{-1} and 10 mg·kg^{-1}) alone attenuated the markers of hepatic I/R injury and potentiated the effect of HU-910, when given in combination (Figure 3). At higher doses (above 10 mg·kg^{-1}), SR144528 alone did attenuate the hepatic I/R injury (data not shown).

HU-910 treatment given immediately after the induction of the ischaemia (Figure 4A) or 1 h following the reperfusion (Figure 4B) still attenuated, although to a lesser extent than in the pretreatment experiments, the hepatic injury measured at 6 h of reperfusion.

HU-910 improves I/R-induced histological damage

Haematoxylin and eosin staining of representative liver sections after 24 h of reperfusion showed (Figure 5) that I/R induced marked coagulation necrosis (lighter areas, with marked inflammatory cell infiltration), which was dramatically reduced and became more focal in mice treated with HU-910 (3 mg·kg^{-1} i.p.). Pretreatment with the CB_2 receptor antagonist/inverse agonist SR144528 (3 mg·kg^{-1} i.p.) almost completely prevented the protective effect of HU-910, as seen on liver histology. Vehicle or HU-910 treatment in the sham animals had no effect on liver histopathology. A similar histological profile was seen throughout the group (n = 3–5 mice).

HU-910 attenuates the marked neutrophil infiltration induced by I/R

Neutrophils are important mediators of the delayed tissue injury following I/R. Most of the hepatic neutrophil infiltration is known to occur between 6 and 24 h of reperfusion (Abe *et al.*, 2009; Mukhopadhyay *et al.*, 2011b). An indicator of neutrophil infiltration is the tissue MPO activity. In sham-treated mice, MPO staining was barely detectable (Figure 6). In contrast, there was a marked increase in infiltrating MPO-positive immune cells (brown staining) after 24 h of reperfusion in vehicle treated animals, which was significantly attenuated by HU-910 3 mg·kg^{-1} i.p. (Figure 6). Pretreatment with the CB_2 receptor antagonist SR144528 (3 mg·kg^{-1} i.p.) almost completely prevented the effect of HU-910 seen on hepatic neutrophil infiltration. Vehicle or HU-910 treatment in the sham animals had no effect on liver MPO staining. A similar histological profile was seen throughout the group (n = 3–5).

HU-910 attenuates the hepatic pro-inflammatory chemokine, cytokine and adhesion molecule expression induced by I/R

I/R greatly increased the expression of mRNA of pro-inflammatory chemokines CCL3, CXCL2 and CCL2 (Figure 7A–C), the pro-inflammatory cytokine TNF-α (Figure 8A) and the adhesion molecule ICAM-1 (Figure 8B) in liver tissue as documented by real-time PCR, which was attenuated by HU-910 (3 mg·kg^{-1} i.p.) given before the ischaemia (Figures 7 and 8). Hepatic TNF-α, CCL3, CXCL2

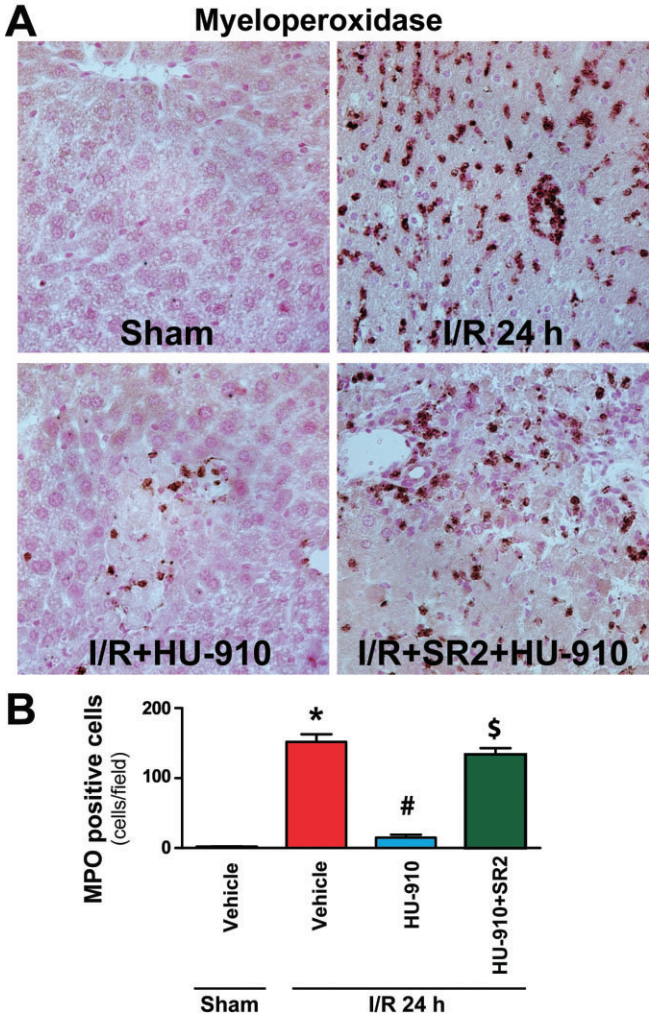

Figure 6

HU-910 attenuates the ischaemia/reperfusion (I/R)-induced increased neutrophil infiltration. (A) Myeloperoxidase (MPO) staining (brown) of representative liver sections of sham mice treated with vehicle (Sham), and mice exposed to 1 h of ischaemia followed by 24 h of reperfusion treated with vehicle (I/R) or HU-910 3 mg·kg^{-1} (I/R + HU-910) or combined HU-910 3 mg·kg^{-1} and SR144528 (SR2; 3 mg·kg^{-1}; I/R + SR2 + HU-910) given before ischaemia. Slides were counterstained by nuclear fast red. Note the marked attenuation of the MPO positive neutrophil infiltration (brown staining) characteristic of I/R 24 h by HU-910, which was largely attenuated by SR144528 pretreatment. At this dose SR144528 alone had no effect on I/R-induced increased MPO positive cell infiltration; neither HU-910 nor SR144528 had effects on normal livers in which the MPO positive infiltrating immune cells were practically absent (data not shown). Images are 400 × magnification. A similar histological profile was seen in three to five livers per treatment group. (B) Quantification of MPO staining in the liver sections. MPO positive cells were counted at 400 × magnification and expressed as MPO positive cells/field. Results are mean ± SEM, $n = 4$. *$P < 0.05$ vehicle in sham versus I/R 24 h; #$P < 0.05$ I/R 24 h versus I/R + HU910 ± SR2; \$$P < 0.05$ I/R + HU910 versus I/R + HU910 + SR2.

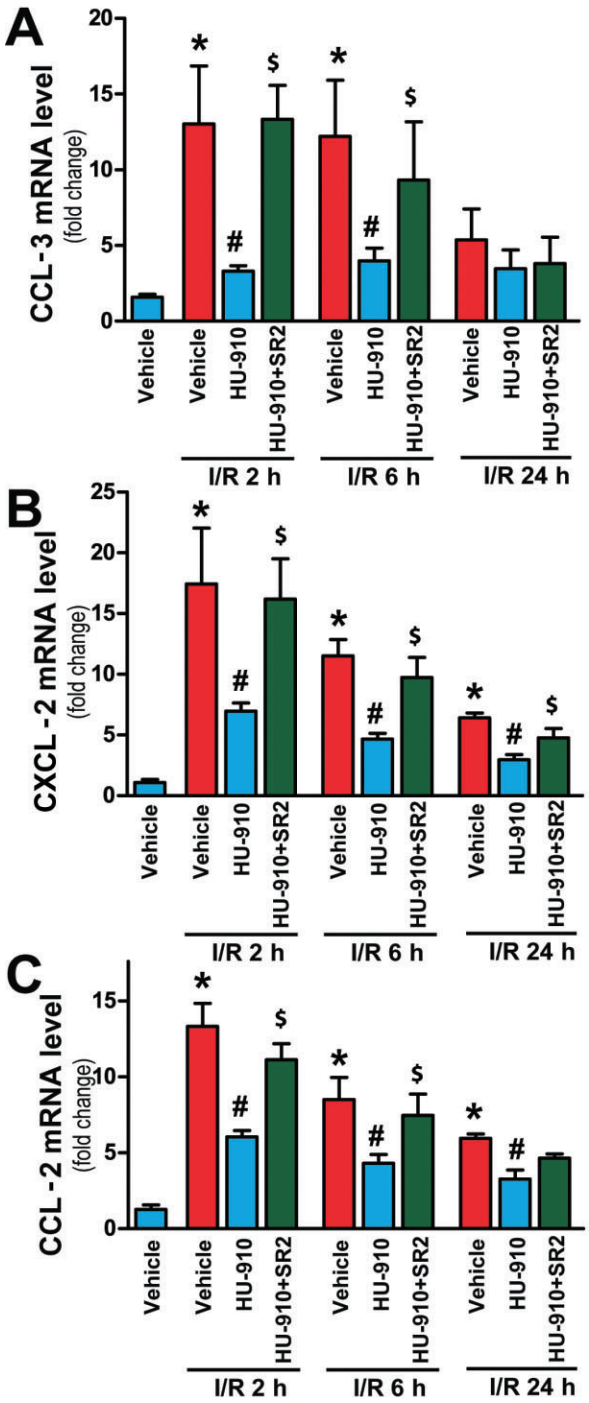

Figure 7

HU-910 attenuates the ischaemia/reperfusion (I/R)-induced acute pro-inflammatory chemokine response in the liver. Real-time PCR shows significant increase of hepatic pro-inflammatory chemokine CCL3 (A), CXCL2 (B), CCL2 (C) mRNA levels at 2 h of reperfusion (I/R 2 h), and a gradual decrease by 6 and 24 h (I/R 6 h and I/R 24 h). Treatment with HU-910 at 3 mg·kg^{-1} before ischaemia significantly attenuates the I/R-induced increased levels of these pro-inflammatory markers. This effect could be largely prevented by pretreatment with SR144528 (SR2; 3 mg·kg^{-1}). Results are mean ± SEM, n = 7–8. *P < 0.05 vehicle versus I/R 2, 6 or 24 h; #P < 0.05 I/R 2, 6 or 24 h versus corresponding I/R + HU-910 ± SR2 mice; \$$P$ < 0.05 I/R + HU-910 versus corresponding I/R + HU-910 + SR2.

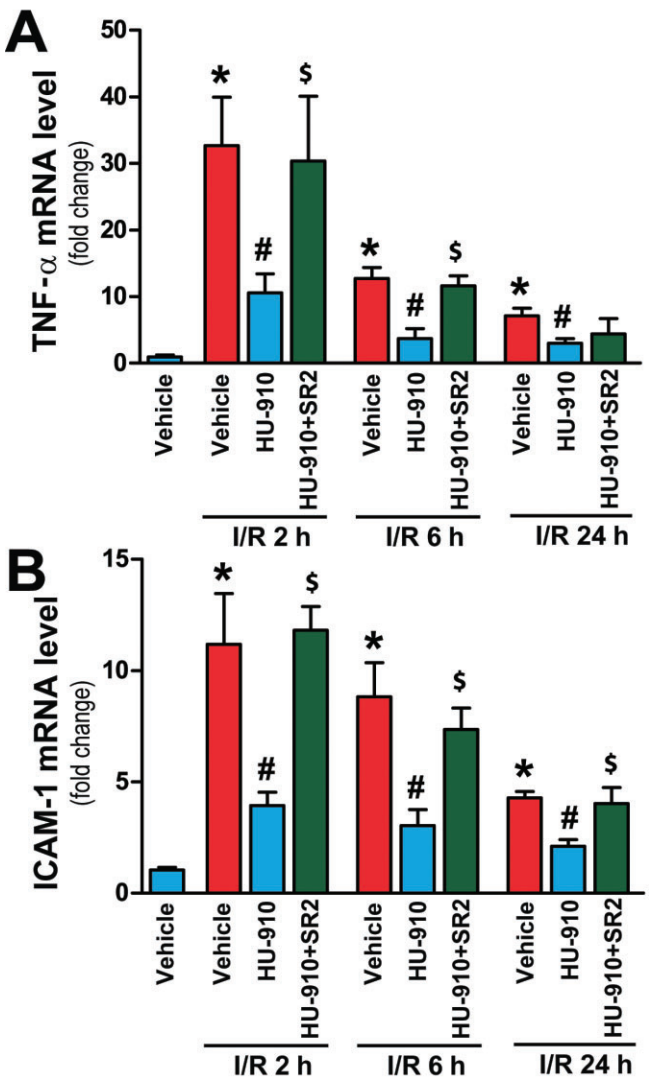

Figure 8

HU-910 attenuates the ischemia/reperfusion (I/R)-induced acute pro-inflammatory cytokine and adhesion molecule response in the liver. Real-time PCR shows significant increase of pro-inflammatory cytokine TNF-α (A), and adhesion molecule ICAM-1 (B) mRNA level at 2 h of reperfusion (I/R 2 h), and a gradual decrease by 6 h and 24 h (I/R 6 h and I/R 24 h). Treatment with HU-910 at 3 mg·kg^{-1} before ischaemia significantly attenuates the I/R-induced increased levels of these pro-inflammatory markers. This effect could be largely prevented by pretreatment with SR144528 (SR2; 3 mg·kg^{-1}) Results are mean ± SEM, n = 6–8/groups. *P < 0.05 vehicle versus I/R 2, 6 or 24 h; #P < 0.05 I/R 2, 6 or 24 h versus corresponding I/R + HU-910 ± SR2 mice; \$$P$ < 0.05 I/R + HU-910 versus corresponding I/R + HU-910 + SR2.

and CCL2 and ICAM-1 mRNA (part of the acute inflammatory response orchestrated by activated Kupffer and endothelial cells) were maximal at 2 h of reperfusion (Figures 7 and 8) and decreased by 24 h of reperfusion almost to control levels. HU-910 given before ischaemia significantly decreased the peak values of CCL3, CXCL2 and CCL2 (Figure 7A–C), TNF-α (Figure 8A) and ICAM-1 (Figure 8B) mRNA in liver. These

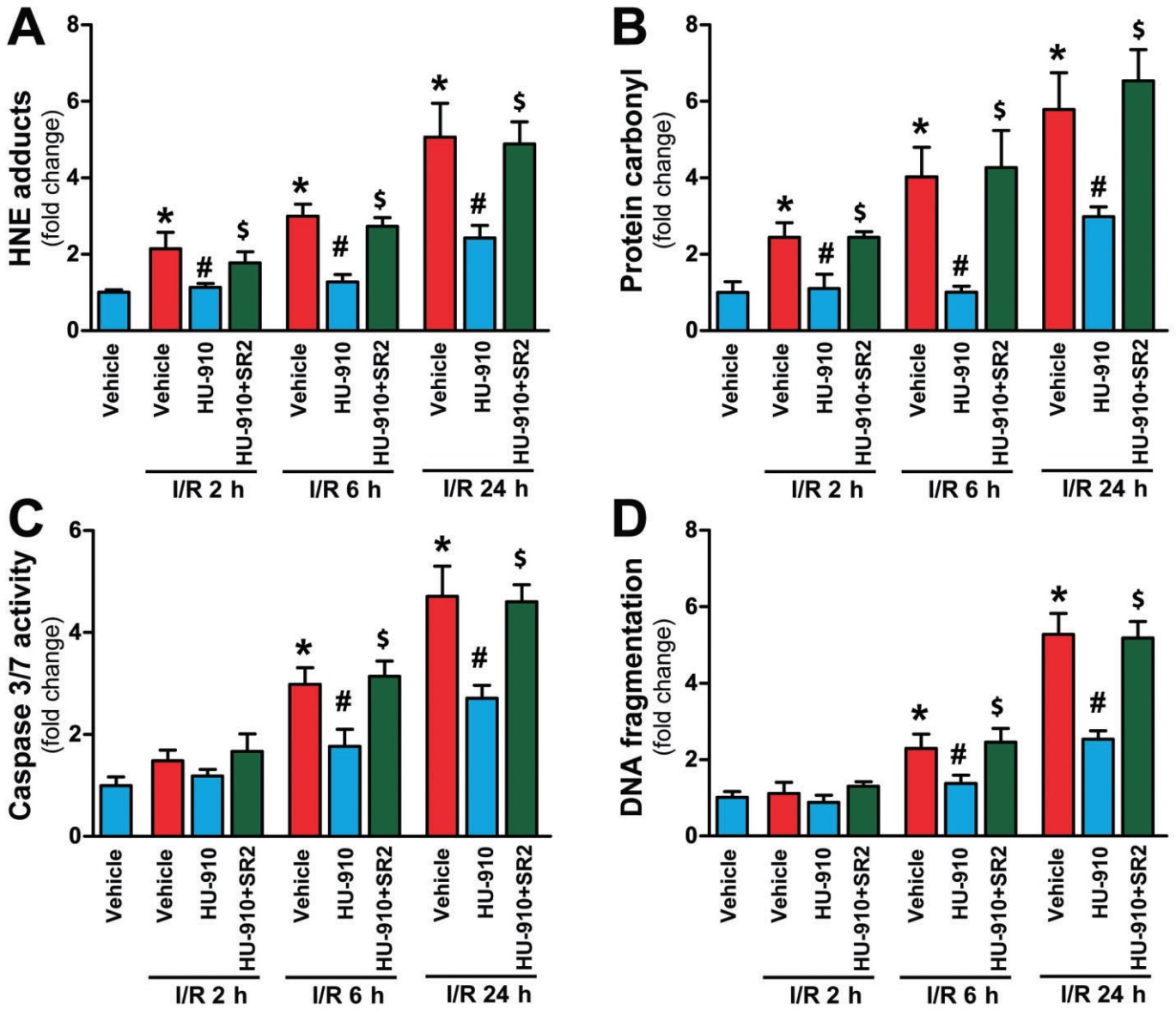

Figure 9

HU-910 attenuates the ischaemia/reperfusion (I/R)-induced increased oxidative stress and apoptotic cell death. (A) HNE adducts (a marker for lipid peroxidation/oxidative stress); (B) Oxidative modification of proteins measured by carbonyl adducts; (C,D) hepatic caspase 3/7 activity and DNA fragmentation (markers of apoptosis). All these markers show time-dependent increases following I/R injury peaking at 24 h of reperfusion. 3 mg·kg^{-1} HU-910 treatment before ischaemia significantly attenuates these increases, which were mostly prevented by pretreatment with SR144528 (SR2; 3 mg·kg^{-1}). Results are mean ± SEM, n = 4–10/groups. *P < 0.05 vehicle versus I/R 2, 6 or 24 h; #P < 0.05 I/R 2, 6 or 24 h versus corresponding I/R + HU-910 ± SR2 mice; \$$P$ < 0.05 I/R + HU-910 versus corresponding I/R + HU-910 + SR2.

effects could be largely attenuated by pretreatment with the CB_2 receptor antagonist SR144528 (3 mg·kg^{-1} i.p.) (Figures 7 and 8).

HU-910 decreases the increased oxidative stress and hepatocyte cell death induced by I/R

The rate of oxidative posttranslational modification of proteins and lipid peroxidation was negligible in sham livers, as indicated by the low level of carbonyl adducts and HNE. We found a time-dependent increase in HNE peaking at 24 h of reperfusion, which was significantly attenuated by HU-910 (3 mg·kg^{-1} i.p.) (Figure 9A). Hepatic content of carbonyl adducts also peaked at 24 h of reperfusion, which was significantly attenuated by HU-910 (3 mg·kg^{-1} i.p.) (Figure 9B).

Apoptotic cell death in the liver following I/R was evaluated by monitoring caspase 3/7 activity and DNA fragmentation (Figure 9C,D). All markers of cell death in the liver were markedly increased at 24 h of reperfusion following 60 min of ischaemia. HU-910 (3 mg·kg^{-1} i.p.) significantly attenuated the increase of both markers (Figure 9C,D).

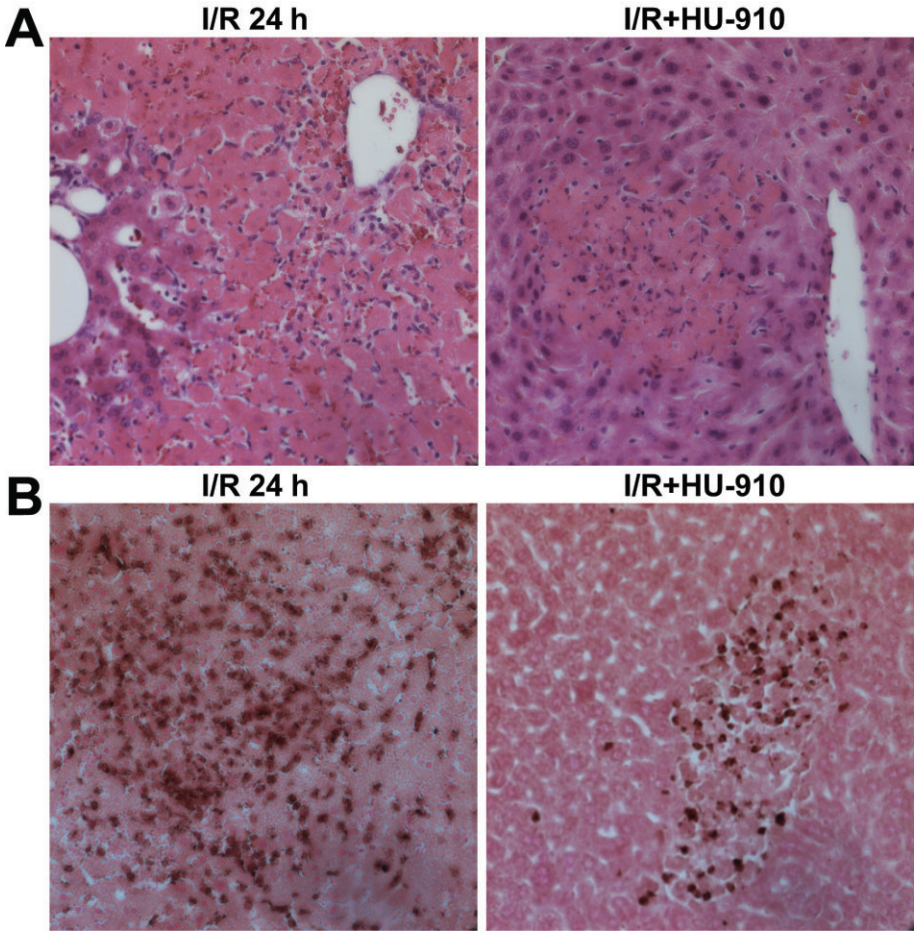

Figure 10

HU-910 treatment (administered after ischaemia) attenuates histological damage at 24 h following ischaemia. (A) Haematoxylin and eosin staining of representative liver sections of mice exposed to 1 h of ischaemia followed by 24 h of reperfusion treated right after the induction of the ischaemia with vehicle [ischaemia/reperfusion (I/R) 24 h] or HU-910 at 3 mg·kg^{-1} (I/R + HU-910). Images are at 400 × magnification. A similar histological profile was seen in three to five livers/group. (B) Myeloperoxidase (MPO) staining (brown) of representative liver sections of mice exposed to 1 h of ischaemia followed by 24 h of reperfusion treated immediately after the induction of the ischaemia with vehicle (I/R 24 h) or HU-910 at 3 mg·kg^{-1} (I/R + HU-910). Images are at 400 × magnification. A similar histological profile was seen in three to five livers per treatment group.

All effects of HU-910 on oxidative and cell death markers were largely prevented by the CB_2 receptor antagonist SR144528 (3 mg·kg^{-1} i.p.) (Figure 9A–D).

HU-910 treatment (administered after ischaemia) attenuates histological damage, oxidative stress, cell death and acute pro-inflammatory response following hepatic ischaemia-reperfusion injury

I/R induced marked coagulation necrosis (lighter areas, with marked inflammatory cell infiltration), which was dramatically reduced and became more focal in mice treated with HU-910 (3 mg·kg^{-1} given immediately after the ischaemic period) (Figure 10A). HU-910 also attenuated the observed increase in infiltrating MPO-positive immune cells (brown staining) after 24 h of reperfusion in vehicle-treated animals (Figure 10B), and also the markers of oxidative stress (protein carbonyl, HNE) and apoptotic cell death (caspase 3/7 activity and DNA fragmentation) (Figure 11A–D).

Furthermore, HU-910 (3 mg·kg^{-1}; administered immediately after the ischaemic period) was also able to attenuate the increased expression of mRNA of CCL3, CXCL2 and CCL2 (Figure 12A–C), of the adhesion molecule ICAM-1 (Figure 12D) and of TNF-α (Figure 12E), after 6 h of reperfusion.

Only the higher dose of HU-910 (10 mg·kg^{-1}) was able to improve the histopathological injury and attenuate the neutrophil infiltration when it was given 1 h after the ischaemic period (data not shown).

HU-910 attenuates the TNF-α production in mouse Kupffer cells induced by LPS

Because the acute inflammatory response in the liver is orchestrated mainly by activated Kupffer and endothelial cells, which express CB_2 receptors (Rajesh *et al.*, 2007; Hall

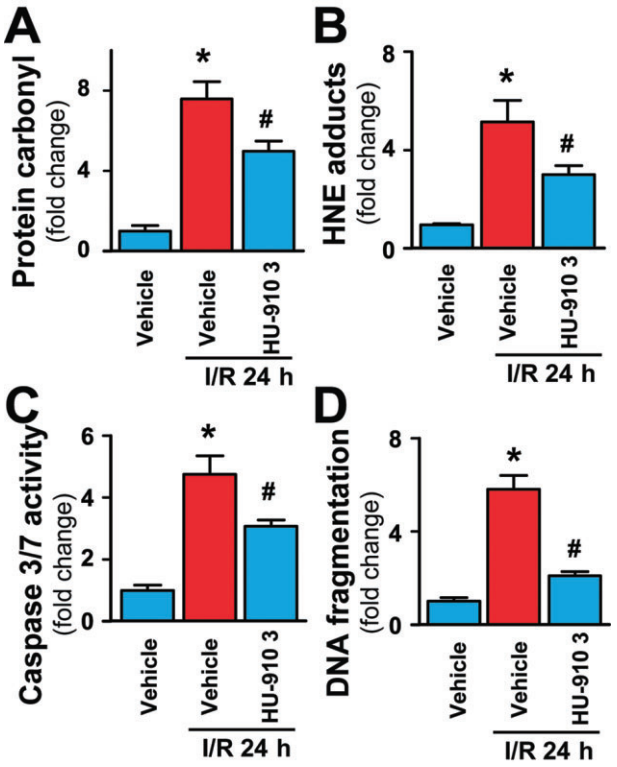

Figure 11

HU-910 treatment (administered after ischaemia) attenuates oxidative stress and cell death at 24 h following ischaemia. Ischaemia/reperfusion (I/R) induced significant increases at 24 h of reperfusion in hepatic oxidative stress markers [protein carbonyl (A) and HNE (B)] and apoptosis [caspase 3/7 activity (C) and DNA fragmentation (D)], which were attenuated by HU-910 3 mg·kg^{-1} given right after the induction of ischaemia. Results are mean ± SEM, n = 5–6. *P < 0.05 vehicle versus I/R 24 h; #P < 0.05 I/R 24 h versus corresponding I/R + HU-910.

et al., 2010; Pacher and Mechoulam, 2011), we also tested the concentration-dependent effect of HU-910 on inflammatory responses of these cell types. Kupffer cells were isolated from mice as described in the Methods section, and then treated with bacterial LPS (100 ng·mL^{-1}) in the presence or absence of HU-910 (10 nM–10 μM), and the CB_2 receptor antagonist SR144528 (1–6 μM). As shown in Figure 13, LPS treatment drastically enhanced TNF-α production in Kupffer cells, which was attenuated in a concentration-dependent manner by HU-910. The effect of 3 μM HU-910 was largely prevented by pretreatment with the CB_2 receptor antagonist SR144528 at 1 μM. Interestingly, higher concentrations of SR144528 by themselves attenuated the TNF-α production induced by LPS, indicating a non-specific effect at these concentrations. Addition of vehicle, HU-910 (10 μM) or SR144528 (1 μM) had no effect on the negligible baseline TNF-α levels in unstimulated Kupffer cells.

HU-910 attenuates the adhesion molecules expression in HLSEC induced by TNF-α

Treatment of HLSEC cells with TNF-α (50 ng·mL^{-1}) for 6 h markedly enhanced the production of the adhesion molecules, ICAM-1 and VCAM-1 (Figure 14). This was inhibited when cells were treated with HU-910 (10 nM–3 μM). The effect of HU-910 (3 μM) was largely attenuated by pretreatment with the CB_2 receptor antagonist SR144528 at 1 μM. Vehicle, HU-910 (3 μM) or SR144528 (1 μM) had no effect on baseline TNF-α levels in unstimulated HLSEC.

Discussion

In the current study, using various *in vitro* assays we have demonstrated that HU-910 was a potent agonist of CB_2 receptors. In these *in vitro* assays, the pharmacological properties of HU-910 on CB_2 receptors were similar to those of HU-308 (Hanus *et al.*, 1999) and JWH-133 (Huffman, 2005; Pertwee, 2005). However, HU-910 had greater *in vivo* efficacy compared with the two earlier CB_2 receptor agonists, by at least one order of magnitude, in terms of attenuating acute and delayed inflammatory response and interrelated oxidative stress and cell death in a murine model of hepatic I/R (Batkai *et al.*, 2007; Rajesh *et al.*, 2007), which is at least in part mediated by activation of CB_2 receptors. We also showed that HU-910 attenuated LPS-triggered TNF-α production in isolated Kupffer cells, and the expression of adhesion molecules in primary HLSEC stimulated with TNF-α.

I/R injury in the liver is a common complication of prolonged surgical procedures, liver transplantation and circulatory shock. The initial damage is inflicted by a sequence of events, when the liver is transiently deprived of its blood supply followed by reoxygenation. These include acute generation of reactive oxygen and nitrogen species from the activation of various cellular and subcellular sources, such as xanthine oxido-reductases and mitochondria (Engerson *et al.*, 1987; Pacher *et al.*, 2006)] during the early reperfusion leading to increased lipid peroxidation, oxidative modification of key proteins involved in cell survival or death, energy metabolism or energy supply (Moon *et al.*, 2008), and oxidative DNA damage (Gero and Szabo, 2006; Jaeschke, 2003). The first line of defence against tissue injury is provided by the endothelial cells which respond to oxidative stress by activation, resulting in release of various inflammatory mediators and increased expression of adhesion molecules. In concert with the endothelial cell activation, the resident macrophages of the liver, the Kupffer cells, are also rapidly activated by reperfusion, generating large amounts of pro-inflammatory chemokines and cytokines which prime and facilitate the recruitment of neutrophils and other inflammatory cells into the liver vasculature upon reperfusion. The inflammatory cells attached to the activated endothelium release more reactive oxidants and pro-inflammatory mediators resulting in endothelial dysfunction and disruption of the endothelial barrier. Through the damaged endothelium, inflammatory cells can transmigrate to the parenchyma and attach to and damage hepatocytes by releasing proteolytic enzymes and oxidants, ultimately leading to cell death (both apoptotic and necrotic) and organ failure (Jaeschke, 2006; Pacher and Hasko, 2008).

Consistent with earlier reports (Moon *et al.*, 2008; Abe *et al.*, 2009; Mukhopadhyay *et al.*, 2011b), we found that the acute inflammatory response in our hepatic I/R model peaked between 2 and 6 h of reperfusion, with marked increases (up

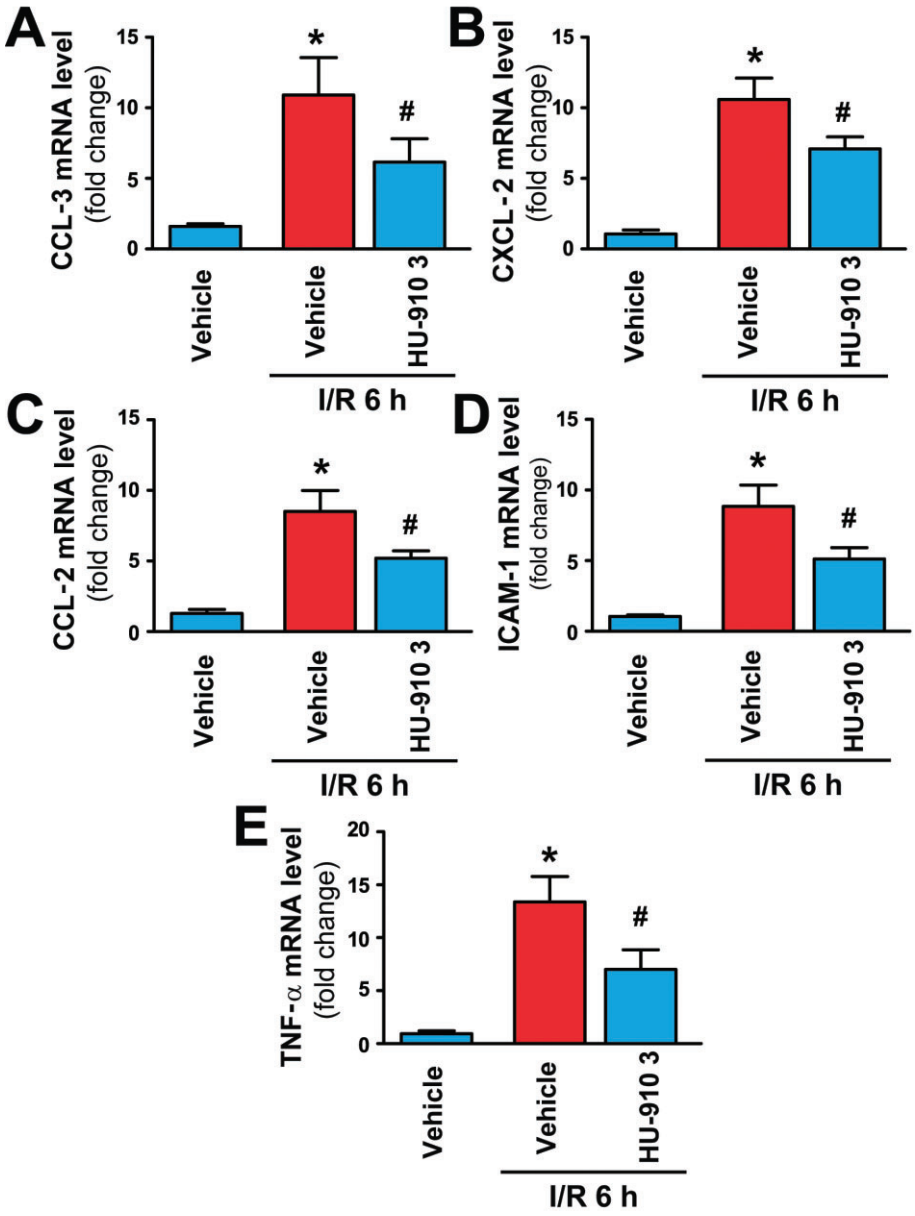

Figure 12

HU-910 (administered after ischaemia) attenuates the ischaemia/reperfusion (I/R)-induced pro-inflammatory response in the liver at 6 h following ischaemia. Real-time PCR shows significant increase of mRNA for chemokines CCL3 (A), CXCL2 (B), CCL2 (C), adhesion molecule ICAM-1 (D), and pro-inflammatory cytokine TNF-α (E) at 6 h of reperfusion (I/R 6 h). Treatment with HU-910 3 $mg \cdot kg^{-1}$ given immediately after the induction of the ischaemia significantly attenuates the I/R-induced increased levels of these inflammatory markers. Results are mean ± SEM, $n = 6–8$. *$P < 0.05$ vehicle versus I/R 6 h; #$P < 0.05$ I/R 6 h versus corresponding I/R + HU-910.

to over 30-fold) in mRNA for TNF-α, CCL3, CXCL2 and CCL2 and ICAM-1 in liver, which declined thereafter by 24 h of reperfusion (Moon *et al.*, 2008; Abe *et al.*, 2009; Mukhopadhyay *et al.*, 2011b). The peak hepatocyte necrosis occurred at 6 h of reperfusion (indicated by peak elevations of serum ALT and AST levels), which gradually returned close to normal levels by 24 h of reperfusion, indicating that the predominant type of cell death at the earlier time points of reperfusion is necrotic. As the chronic inflammatory reaction develops (from 12 h of reperfusion), the histological picture of post-ischaemic hepatic morphology at 24 h of reperfusion is characterized by marked coagulation necrosis (lighter areas) with massive inflammatory MPO-positive neutrophil cell infiltration (Figures 5, 6 and 10), which parallels with increases in oxidative stress (indicated by HNE and protein carbonyl levels) and apoptotic cell death (caspase 3/7 activity and DNA fragmentation).

There is considerable interest in the development of novel selective CB_2 receptor agonists, which lack the psychoactive properties of CB_1 receptor agonists, for the treatment of various inflammatory and other disorders (Pacher and Mechoulam, 2011). As mentioned earlier, numerous recent

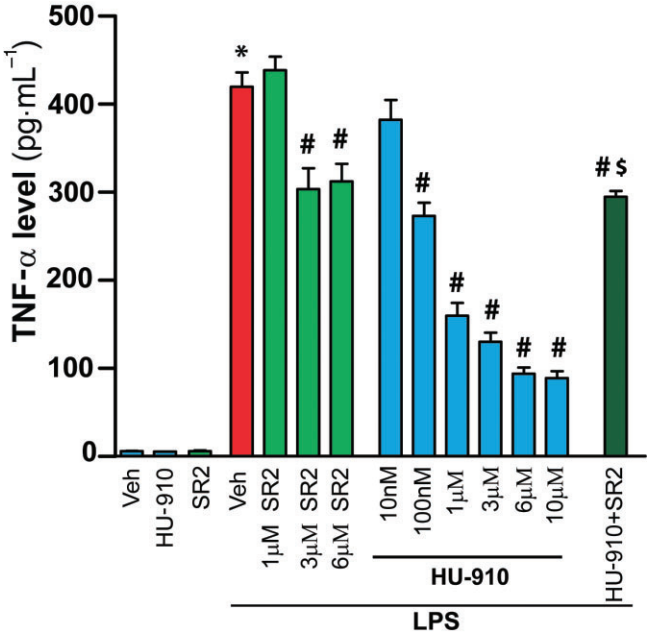

Figure 13

HU-910 attenuates lipopolysaccharide (LPS)-induced TNF-α secretion in Kupffer cells. HU-910 (10 nM-10 μM) attenuates LPS-induced TNF-α secretion of Kupffer cells in a concentration-dependent manner. The CB_2 receptor antagonist SR144528 (SR2; 1 μM) attenuates the effects of 3 μM of HU-910. At higher concentrations SR144528 alone also shows some inhibitory effect on LPS-induced TNF-α secretion. Results are mean ± SEM, $n = 6$. *$P < 0.05$ vehicle (Veh) versus LPS (100 ng·mL^{-1}); #$P < 0.05$ LPS versus LPS + HU-910 ± SR2; \$$P < 0.05$ LPS + HU-910 3 μM versus LPS + HU-910 ± SR2.

studies using potent CB_2 receptor agonists and/or knockout mice have provided compelling evidence that CB_2 receptor activation is protective against myocardial (Montecucco *et al.*, 2009), cerebral (Zhang *et al.*, 2007; 2009a,b; Murikinati *et al.*, 2010) and hepatic (Batkai *et al.*, 2007; Rajesh *et al.*, 2007) I/R injuries by decreasing the endothelial cell activation/inflammatory response [e.g. expression of adhesion molecules, secretion of chemokines (Batkai *et al.*, 2007; Rajesh *et al.*, 2007)], and by attenuating the leukocyte chemotaxis, rolling, adhesion to endothelium, activation and transendothelial migration, and interrelated oxidative/nitrosative damage (Pacher and Hasko, 2008; Zhang *et al.*, 2009a).

In agreement with the above mentioned reports, we found that the peak elevations of serum liver transaminases (ALT/AST) occurred at 6 h following reperfusion (Figure 2), and were dose-dependently (1–10 mg·kg^{-1}) attenuated by HU-910 given before the induction of I/R. 1 mg·kg^{-1} of HU-910 was sufficient to achieve similar or greater protection (as shown by decreases in I/R-induced elevated serum ALT/AST) during hepatic I/R injury, compared with the effects reported with HU-308 and JWH-133 at doses of 10 and 20 mg·kg^{-1} (Batkai *et al.*, 2007; Rajesh *et al.*, 2007) respectively. The selected optimal dose (3 mg·kg^{-1}) for mechanistic studies significantly decreased tissue oxidative stress (HNE and carbonyl adducts), ameliorated acute and chronic hepatic inflammatory responses (CCL3, CXCL2 and CCL2,

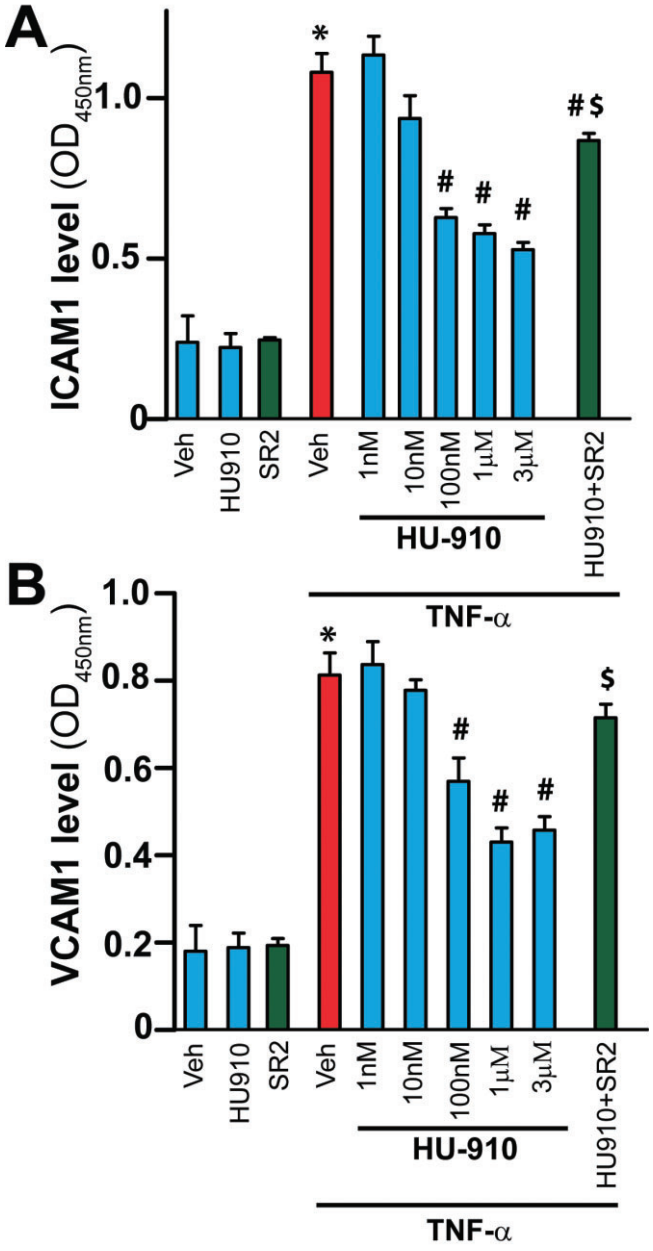

Figure 14

HU-910 attenuates the TNFα-induced adhesion molecules expression in human liver sinusoidal endothelial cells (HLSEC). Treatment of HLSEC cells with 50 ng·mL^{-1} TNF-α for 6 h, markedly enhances the expression of adhesion molecules such as ICAM-1 and VCAM-1. HU-910 concentration-dependently (1 nM-10 μM) attenuates this enhanced expression of adhesion molecules ICAM-1 (A) and VCAM-1 (B). The CB_2 receptor antagonist SR144528 (SR2; 1 μM) attenuates this effect of HU-910 on adhesion molecule expression. Results are mean ± SEM, $n = 5$–6/group. *$P < 0.05$ vehicle (Veh) versus TNF-α (50 ng·mL^{-1}); #$P < 0.05$ TNF-α versus TNF-α + HU-910 ± SR2; \$$P < 0.05$ TNF-α + HU-910 3 μM versus TNF-α + HU-910 ± SR2.

TNF-α, ICAM-1/CD54 mRNA levels and tissue neutrophil infiltration), and both necrotic (ALT/AST levels and coagulation necrosis) and apoptotic (caspase 3/7 activity, DNA fragmentation) cell death at various relevant (2, 6 and/or 24 h

following ischaemia) time points. Furthermore, HU-910 concentration-dependently attenuated theLPS-triggered TNF-α production in isolated Kupffer cells, and expression of adhesion molecules in primary HLSEC stimulated with TNF-α. These beneficial effects of HU-910 (3 $mg \cdot kg^{-1}$) were largely attenuated by the CB_2 receptor antagonist/inverse agonist SR144528 (3 $mg \cdot kg^{-1}$), which had no significant effect on I/R injury or the inflammatory response by itself; indicating that these effects were, at least in part, mediated by CB_2 receptor activation. The fact that the effect of the highest dose of HU-910 used (10 $mg \cdot kg^{-1}$) was only partially attenuated by SR144528 could indicate some protective effects of the compound unrelated to CB_2 receptor activation. However, the absence of the complete reversal can also be explained by the existence of non-specific anti-inflammatory effects of SR144528. This is also supported by aggravated hepatic I/R injury in CB_2 knockout mice compared with their wild-type littermates, which cannot be mimicked by pretreatment of mice exposed to hepatic I/R with SR144528 at 3 $mg \cdot kg^{-1}$ i.p. However, this dose of SR144528 was able to attenuate the protective effect of CB2 agonist JWH-133 given at 20 $mg \cdot kg^{-1}$ i.p. (Batkai *et al.*, 2007). Pretreatment with the CB_1 receptor antagonist SR141716 (rimonabant) did not prevent the protective effect of HU-910 on liver necrosis; in fact, it reduced the serum ALT/AST levels by itself, an effect more pronounced when HU-910 was given in combination with SR141716. The protective effect of the CB_1 receptor antagonist/inverse agonist on hepatic I/R injury described in our current report is in agreement with the protection observed in a model of rat liver I/R complicated with endotoxaemia (Caraceni *et al.*, 2009) and in other models of I/R injury (Pacher and Hasko, 2008; Zhang *et al.*, 2009b), as well as with the proposition of combining CB_1 antagonists with CB_2 receptor agonists for the treatment of reperfusion injury (Pacher and Hasko, 2008; Zhang *et al.*, 2009b). Importantly, some of the protective effects of HU-910 also persisted when it was administered either at the beginning of the reperfusion or 1 h after the ischaemic episode (see Figures 4 and 10–12 and *Results*).

Collectively, our results suggest that HU-910 is a novel CB_2 receptor agonist which may exert protective effects in various diseases associated with inflammation and tissue injury. Its greater efficacy *in vivo*, despite similar pharmacological properties *in vitro* on CB_2 receptors to HU-308 or JWH-133, could be explained, at least in part, by its better penetration to injured tissues, or perhaps some additional beneficial properties unrelated to CB_2 receptor activation, which together with its therapeutic window should be explored in the future studies. The beneficial effects of HU-910 against hepatic I/R injury are particularly exciting, because CB_2 receptors are emerging as important targets in various liver (Lotersztajn *et al.*, 2008; Pacher and Gao, 2008) and other (Pacher and Mechoulam, 2011) diseases and their complications, and may also be crucial in liver regeneration (Teixeira-Clerc *et al.*, 2010).

Acknowledgements

This study was supported by the Intramural Research Program of NIH/NIAAA (to P. P.) and by NIDA grant #9789 (to R. M.). Dr Béla Horváth is a recipient of a Hungarian Research Council Scientific Research Fund Fellowship (OTKA-NKTH-EU MB08-80238). The authors are indebted to Drs George Kunos and Bin Gao for providing key resources and support.

Conflict of interest

Dr R. Mechoulam and L. Magid have submitted a patent on HU-910, other authors have no conflicts to disclose.

References

Abe Y, Hines IN, Zibari G, Pavlick K, Gray L, Kitagawa Y *et al.* (2009). Mouse model of liver ischemia and reperfusion injury: method for studying reactive oxygen and nitrogen metabolites in vivo. Free Radic Biol Med 46: 1–7.

Alexander SPH, Mathie A, Peters JA (2011). Guide to Receptors and Channels (GRAC), 5th Edition. Br J Pharmacol 164 (Suppl. 1): S1–S324.

Batkai S, Osei-Hyiaman D, Pan H, El-Assal O, Rajesh M, Mukhopadhyay P *et al.* (2007). Cannabinoid-2 receptor mediates protection against hepatic ischemia/reperfusion injury. FASEB J 21: 1788–1800.

Caraceni P, Pertosa AM, Giannone F, Domenicali M, Grattagliano I, Principe A *et al.* (2009). Antagonism of the cannabinoid CB-1 receptor protects rat liver against ischaemia-reperfusion injury complicated by endotoxaemia. Gut 58: 1135–1143.

Di Marzo V (2008). Targeting the endocannabinoid system: to enhance or reduce? Nat Rev Drug Discov 7: 438–455.

Dominianni SJ, Ryan CW, DeArmitt CW (1977). Synthesis of 5-(tert-alkyl)resorcinols. J Org Chem 42: 344–346.

Engerson TD, McKelvey TG, Rhyne DB, Boggio EB, Snyder SJ, Jones HP (1987). Conversion of xanthine dehydrogenase to oxidase in ischemic rat tissues. J Clin Invest 79: 1564–1570.

Ferdinandy P, Schulz R (2003). Nitric oxide, superoxide, and peroxynitrite in myocardial ischaemia-reperfusion injury and preconditioning. Br J Pharmacol 138: 532–543.

Gero D, Szabo C (2006). Role of the peroxynitrite-poly (ADP-ribose) polymerase pathway in the pathogenesis of liver injury. Curr Pharm Des 12: 2903–2910.

Hall D, Poussin C, Velagapudi VR, Empsen C, Joffraud M, Beckmann J *et al.* (2010). Peroxisomal and microsomal lipid pathways associated with resistance to hepatic steatosis and reduced pro-inflammatory state. J Biol Chem 285: 31011–31023.

Hanus L, Breuer A, Tchilibon S, Shiloah S, Goldenberg D, Horowitz M *et al.* (1999). HU-308: a specific agonist for CB_2, a peripheral cannabinoid receptor. Proc Natl Acad Sci U S A 96: 14228–14233.

Huffman JW (2005). CB_2 receptor ligands. Mini Rev Med Chem 5: 641–649.

Jaeschke H (2003). Role of reactive oxygen species in hepatic ischemia-reperfusion injury and preconditioning. J Invest Surg 16: 127–140.

Jaeschke H (2006). Mechanisms of Liver Injury. II. Mechanisms of neutrophil-induced liver cell injury during hepatic ischemia-reperfusion and other acute inflammatory conditions. Am J Physiol Gastrointest Liver Physiol 290: G1083–G1088.

Julien B, Grenard P, Teixeira-Clerc F, Van Nhieu JT, Li L, Karsak M *et al*. (2005). Antifibrogenic role of the cannabinoid receptor CB_2 in the liver. Gastroenterology 128: 742–755.

Liaudet L, Szabo G, Szabo C (2003). Oxidative stress and regional ischemia-reperfusion injury: the peroxynitrite-poly(ADP-ribose) polymerase connection. Coron Artery Dis 14: 115–122.

Lotersztajn S, Teixeira-Clerc F, Julien B, Deveaux V, Ichigotani Y, Manin S *et al*. (2008). CB_2 receptors as new therapeutic targets for liver diseases. Br J Pharmacol 153: 286–289.

Magid L, Goodfellow CE, Srebnik M, Glass M, Alexandrovich A, Hanuš L *et al*. (2010a). Design, synthesis and neuroprotrective potential of novel CB2 receptor selective agonists. 20th Annual Symposium on the Cannabinoids, International Cannabinoid Research Society, Research Triangle Park, NC, USA, Abstracts, p 42.

Magid L, Mechoulam R, Shohami E, Bab I (2010b). Novel arylated camphenes, processes for their preparation and uses thereof. Israeli Patent PCT/IL2010/000970.

Montecucco F, Lenglet S, Braunersreuther V, Burger F, Pelli G, Bertolotto M *et al*. (2009). CB_2 cannabinoid receptor activation is cardioprotective in a mouse model of ischemia/reperfusion. J Mol Cell Cardiol 46: 612–620.

Moon KH, Hood BL, Mukhopadhyay P, Rajesh M, Abdelmegeed MA, Kwon YI *et al*. (2008). Oxidative inactivation of key mitochondrial proteins leads to dysfunction and injury in hepatic ischemia reperfusion. Gastroenterology 135: 1344–1357.

Mukhopadhyay P, Rajesh M, Batkai S, Kashiwaya Y, Hasko G, Liaudet L *et al*. (2009). Role of superoxide, nitric oxide, and peroxynitrite in doxorubicin-induced cell death in vivo and in vitro. Am J Physiol Heart Circ Physiol 296: H1466–H1483.

Mukhopadhyay P, Rajesh M, Batkai S, Patel V, Kashiwaya Y, Liaudet L *et al*. (2010). CB_1 cannabinoid receptors promote oxidative stress and cell death in murine models of doxorubicin-induced cardiomyopathy and in human cardiomyocytes. Cardiovasc Res 85: 773–784.

Mukhopadhyay P, Horvath B, Rajesh M, Matsumoto S, Saito K, Batkai S *et al*. (2011a). Fatty acid amide hydrolase is a key regulator of endocannabinoid-induced myocardial tissue injury. Free Radic Biol Med 50: 179–195.

Mukhopadhyay P, Rajesh M, Horvath B, Batkai S, Park O, Tanchian G *et al*. (2011b). Cannabidiol protects against hepatic ischemia/reperfusion injury by attenuating inflammatory signaling and response, oxidative/nitrative stress, and cell death. Free Radic Biol Med 50: 1368–1381.

Murikinati S, Jüttler E, Keinert T, Ridder DA, Muhammad S, Waibler Z *et al*. (2010). Activation of cannabinoid 2 receptors protects against cerebral ischemia by inhibiting neutrophil recruitment. FASEB J 24: 788–798.

Pacher P, Gao B (2008). Endocannabinoids and liver disease. III. Endocannabinoid effects on immune cells: implications for inflammatory liver diseases. Am J Physiol Gastrointest Liver Physiol 294: G850–G854.

Pacher P, Hasko G (2008). Endocannabinoids and cannabinoid receptors in ischaemia-reperfusion injury and preconditioning. Br J Pharmacol 153: 252–262.

Pacher P, Mechoulam R (2011). Is lipid signaling through cannabinoid 2 receptors part of a protective system? Prog Lipid Res 50: 193–211.

Pacher P, Batkai S, Kunos G (2006). The endocannabinoid system as an emerging target of pharmacotherapy. Pharmacol Rev 58: 389–462.

Pacher P, Beckman JS, Liaudet L (2007). Nitric oxide and peroxynitrite in health and disease. Physiol Rev 87: 315–424.

Pertwee RG (2005). Pharmacological actions of cannabinoids. Handb Exp Pharmacol 168: 1–51.

Rajesh M, Pan H, Mukhopadhyay P, Batkai S, Osei-Hyiaman D, Hasko G *et al*. (2007). Cannabinoid-2 receptor agonist HU-308 protects against hepatic ischemia/reperfusion injury by attenuating oxidative stress, inflammatory response, and apoptosis. J Leukoc Biol 82: 1382–1389.

Rajesh M, Mukhopadhyay P, Batkai S, Mukhopadhyay B, Patel V, Hasko G *et al*. (2009). Xanthine oxidase inhibitor allopurinol attenuates the development of diabetic cardiomyopathy. J Cell Mol Med 13: 2330–2341.

Rajesh M, Mukhopadhyay P, Bátkai S, Patel V, Saito K, Matsumoto S *et al*. (2010). Cannabidiol attenuates cardiac dysfunction, oxidative stress, fibrosis, and inflammatory and cell death signaling pathways in diabetic cardiomyopathy. J Am Coll Cardiol 56: 2115–2125.

Teixeira-Clerc F, Belot MP, Manin S, Deveaux V, Cadoudal T, Chobert MN *et al*. (2010). Beneficial paracrine effects of cannabinoid receptor 2 on liver injury and regeneration. Hepatology 52: 1046–1059.

Zhang M, Martin BR, Adler MW, Razdan RK, Jallo JI, Tuma RF (2007). Cannabinoid CB_2 receptor activation decreases cerebral infarction in a mouse focal ischemia/reperfusion model. J Cereb Blood Flow Metab 27: 1387–1396.

Zhang M, Adler MW, Abood ME, Ganea D, Jallo J, Tuma RF (2009a). CB_2 receptor activation attenuates microcirculatory dysfunction during cerebral ischemic/reperfusion injury. Microvasc Res 78: 86–94.

Zhang M, Martin BR, Adler MW, Razdan RJ, Kong W, Ganea D *et al*. (2009b). Modulation of cannabinoid receptor activation as a neuroprotective strategy for EAE and stroke. J Neuroimmune Pharmacol 4: 249–259.